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PURIFICATION AND PROPERTIES OF 1,2-DEHYDRORETICULINE REDUCTASE FROM *PAPAVER SOMNIFERUM* SEEDLINGS*

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Key Word Index—*Papaver somniferum*; Papaveraceae; 1,2-dehydroreticuline; (*R*)-reticuline; 1,2-dehydroreticuline reductase; purification; characterization; alkaloid biosynthesis; morphinans.

Abstract—1,2-Dehydroreticuline reductase, the NADPH-dependent enzyme which reduces stereospecifically 1,2-dehydroreticuline to (*R*)-reticuline has been discovered in seedlings of the opium poppy (*Papaver somniferum*). The enzyme has been purified to apparent electrophoretic homogeneity by ammonium sulphate precipitation and five subsequent column chromatography steps. The isolated enzyme is a single polypeptide with M_r 30 000 and has a pH optimum at 8.5 and a temperature optimum at 30°. The apparent K_m values for 1,2-dehydroreticuline and NADPH are 10 and 7 μM, respectively. The enzyme mediates the transfer of the pro-*S*-hydride of NADPH to C-1 of 1,2-dehydroreticuline with high substrate specificity; neither 1,2-dehydronorreticuline nor 1,2-dehydrococlaurine are utilized by the enzyme. The enzyme activity is inhibited by (*S*)- and (*R*)-reticuline with I_{50} values of 0.05 and 0.10 mM, respectively. The reductase is a cytosolic enzyme and present only in morphinan alkaloid-containing plants. This highly species-, substrate- and stereospecific enzyme catalyses the provision of (*R*)-reticuline for the formation of morphinan alkaloids that possess also (*R*)-configuration at that chiral centre.

INTRODUCTION

Both the (*S*)- and (*R*)-enantiomeric forms of reticuline have been firmly established in *Papaver somniferum* as the biosynthetic precursors of morphinan-type alkaloids [1, 2]. Of the two enantiomers, it is the (*R*)- form of reticuline that corresponds in absolute stereochemistry to the configuration found at that particular chiral centre in this alkaloid group. Therefore, isomerization of (*S*)-reticuline to its (*R*)-counterpart was postulated [1, 3]. This inversion of configuration was most plausibly explained by the intermediate formation of the 1,2-dehydroreticulinium ion originating from (*S*)-reticuline followed by stereospecific reduction to yield the desired (*R*)-enantiomer [1] (Fig. 1). Support of this proposal was found when synthetic material characterized as 1,2-dehydroreticulinium ion chloride was efficiently incorporated into opium alkaloids [1]. It was not until 1978, however, that Borkowski *et al.* [3] unequivocally showed that 1,2-dehydroreticuline is a naturally occurring compound and that it is incorporated into the morphine alkaloids. These experiments supported fully the intermediacy of 1,2-dehydroreticuline in the isomerization of (*S*)- to (*R*)-reticuline.

The proposed isomerization of reticuline, however, has not been demonstrated at the cell-free level and, therefore, the enzymes involved have not yet been discovered. It is our primary aim to isolate and characterize the enzymes involved in morphinan alkaloid biosynthesis. For this purpose, radioactively labelled [N-$^{14}CH_3$]-1,2-dehydroreticulinium ion was synthesized from [N-$^{14}CH_3$]-(*S*)-reticuline employing the enzyme (*S*)-tetrahydroprotoberberine oxidase [4]. The labelled iminium ion was then fed to five-day-old seedlings of *P. somniferum* through the root system, resulting in good incorporation (3.3%) into thebaine. This experiment demonstrates that the enzymes involved in utilizing 1,2-dehydroreticulinium ion in *P. somniferum* seedlings are metabolically active, prompting us to search for the enzymes which could be involved in the isomerization of (*S*)-reticuline.

The present report describes detection, purification and characterization of the enzyme 1,2-dehydroreticuline reductase which reduces stereospecifically 1,2-dehydroreticuline to (*R*)-reticuline.

RESULTS

Enzyme detection, product identification and enzyme assay

The incubation of [N-$^{14}CH_3$]-1,2-dehydroreticuline with crude proteinaceous extracts of five-day-old *Papaver somniferum* seedlings, in the presence of NADPH at pH 8.5, resulted in the rapid appearance of a radiolabelled product chromatographically indistinguishable from authentic reticuline (Fig. 2). Absolutely no trans-

*A preliminary report on this enzyme has appeared in *Tetrahedron Letters* **31**, 4855 (1990).

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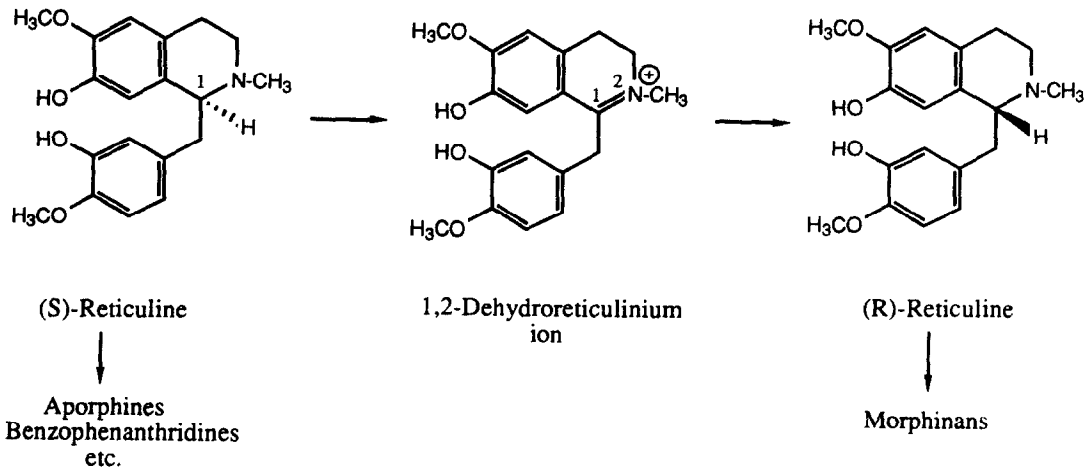

Fig. 1. Proposed isomerization of (*S*)-reticuline to (*R*)-reticuline and their involvement in *Papaver* alkaloid biosynthesis.

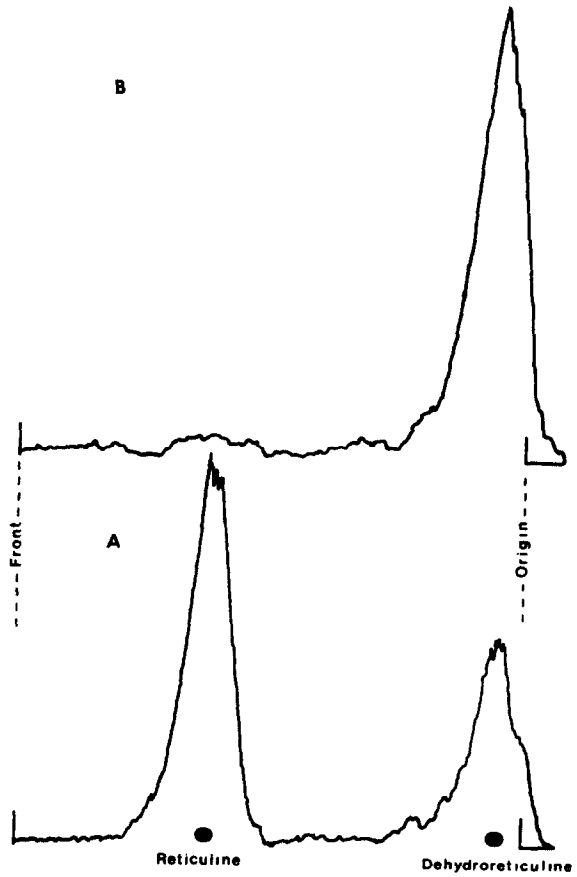

Fig. 2. Radioscans of (A) complete standard assay mixture containing [N-$^{14}CH_3$]-1,2-dehydroreticuline (6.5 μM, 1×10^5 dpm) and (B) a control without NADPH.

formation of the substrate occurred either in the absence of NADPH or in the control (boiled). The product obtained by large-scale (500-fold) incubation was unequivocally identified as reticuline by mass spectral analysis (EI; 70 eV): *m/z* (% relative intensity) = 192 (base peak) (100), 177 (21), 149 (5), 137 (3). The absolute configuration of reticuline was further examined by employing the following three methods. Firstly, the product was analysed by a highly sensitive (*R*)- and (*S*)-reticuline specific radioimmunoassay [5] and was clearly shown to have (*R*)-configuration. (*R*)-Reticuline was rapidly formed during an 8 hr incuabtion period, whereas the (*S*)-enantiomer could not be detected (Fig. 3). Secondly, the CD spectrum of the enzymatically prepared product displayed two negative Cotton effects at 235.8 nm (−0.16)

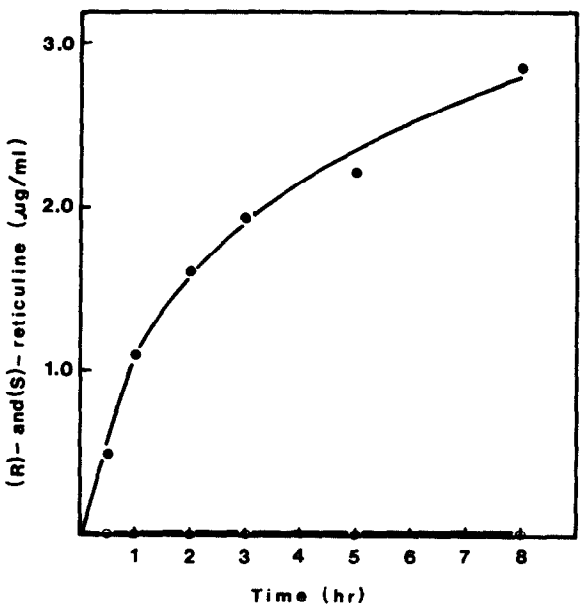

Fig. 3. Time course of product formation in the reaction mixture catalysed by purified 1,2-dehydroreticuline reductase from *P. somniferum*. The product was analysed by a specific radioimmunoassay for (*R*)-reticuline (●) and (*S*)-reticuline (○).

and 290 nm (−0.06). Comparison with authentic (*R*)-reticuline showed beyond doubt that the enzymic product possessed (*R*)-configuration. Thirdly, the highly radiolabelled reticuline metabolite generated from 1,2-dehydroreticuline was not biotransformed to scoulerine by the highly (*S*)-specific berberine bridge enzyme [6].

As already mentioned, the novel enzyme was assayed for catalytic activity by monitoring the transformation of [N-$^{14}CH_3$]-1,2-dehydroreticulinium ion to [N-$^{14}CH_3$]-(*R*)-reticuline. Because of their extreme difference in solubility and, therefore, distribution between aqueous and organic phases, the labelled product could be extracted into toluene while the unreacted radioactive substrate remained in the aqueous phase. More than 95% of the total radioactivity of [N-$^{14}CH_3$]reticuline could be recovered in toluene (1 ml) during the first extraction. Therefore, the radioactivity in the organic phase was taken as a measure of enzyme activity.

Time course of 1,2-*dehydroreticuline reductase activity in* P. somniferum *seedlings*

Using the radioassay system developed, the activity of 1,2-dehydroreticuline reductase in crude enzyme extracts of *P. somniferum* seedlings could be detected easily. The time course of reductase activity in the seedlings was therefore investigated in order to observe deviations during seed generation. The measurable onset of enzyme activity was at day 2 after germination (Fig. 4). The enzyme activity then increased rapidly to reach a maximum value of 22 pkat g^{-1} dry wt at day 4. Thereafter, the activity declined gradually to 14 pkat g^{-1} dry wt at day 10. A similar pattern was also obtained when enzyme activity was expressed as pkat per 10^3 seedlings. To obtain suitable amounts of the reductase for subsequent purification, four- to seven-day-old seedlings were used as an enzyme source.

Enzyme purification

The major portion of the 1,2-dehydroreticuline reductase present in the 10000 *g* supernatant of crude extracts was found in the protein fraction which precipitated at 60–85% saturation with ammonium sulphate. This step removed a large portion (90%) of protein and resulted in a five-fold purification. Upon passage of the resulting protein fraction through Phenyl–Sepharose pre-equilibrated with 5% ammonium sulphate, a significant amount of protein could be eluted whereas reductase activity was retained. The enzyme was eluted with ammonium sulphate-free buffer (Fig. 5A) resulting in an over 15-fold purification step. When the enzyme preparation was passed through DEAE-Sephacel (pH 7.5), the bulk of enzyme activity was retained. The reductase was eluted with a major protein peak (Fig. 5B), and this step gave an additional three-fold purification. Upon Ultrogel-AcA 34 gel filtration of the resulting active enzyme fraction, the reductase activity was found within a trailing shoulder of a major protein peak (Fig. 5C). When the combined active fractions were passed through hydroxyapatite at pH 7.5, about 30% of the protein in the preparation was absorbed. The reductase was eluted as a fairly broad peak (Fig. 5D) with 60% yield giving an additional 1.6-fold purification. The resulting enzyme preparation was finally subjected to Superose 12 gel filtration and the active enzyme fraction was used as the purified preparation.

As summarized in Table 1, the purification method described above led to about 1400-fold purification with 22% recovery of the enzyme activity. The entire purification procedure could be completed within three to four days. The specific 1,2-dehydroreticuline reductase activity of the purified preparations varied from 170 to 220 pkat mg^{-1} protein. The purity of the enzyme preparation was checked by SDS-PAGE. In this system, the enzyme migrated as a single band showing no major protein contamination (Fig. 6).

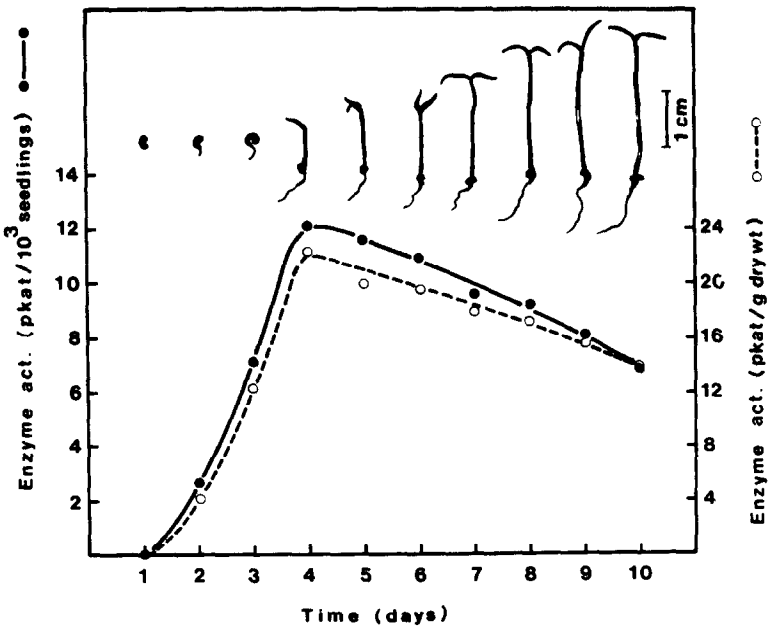

Fig. 4. Time course of 1,2-dehydroreticuline reductase activity in *P. somniferum* seedlings.

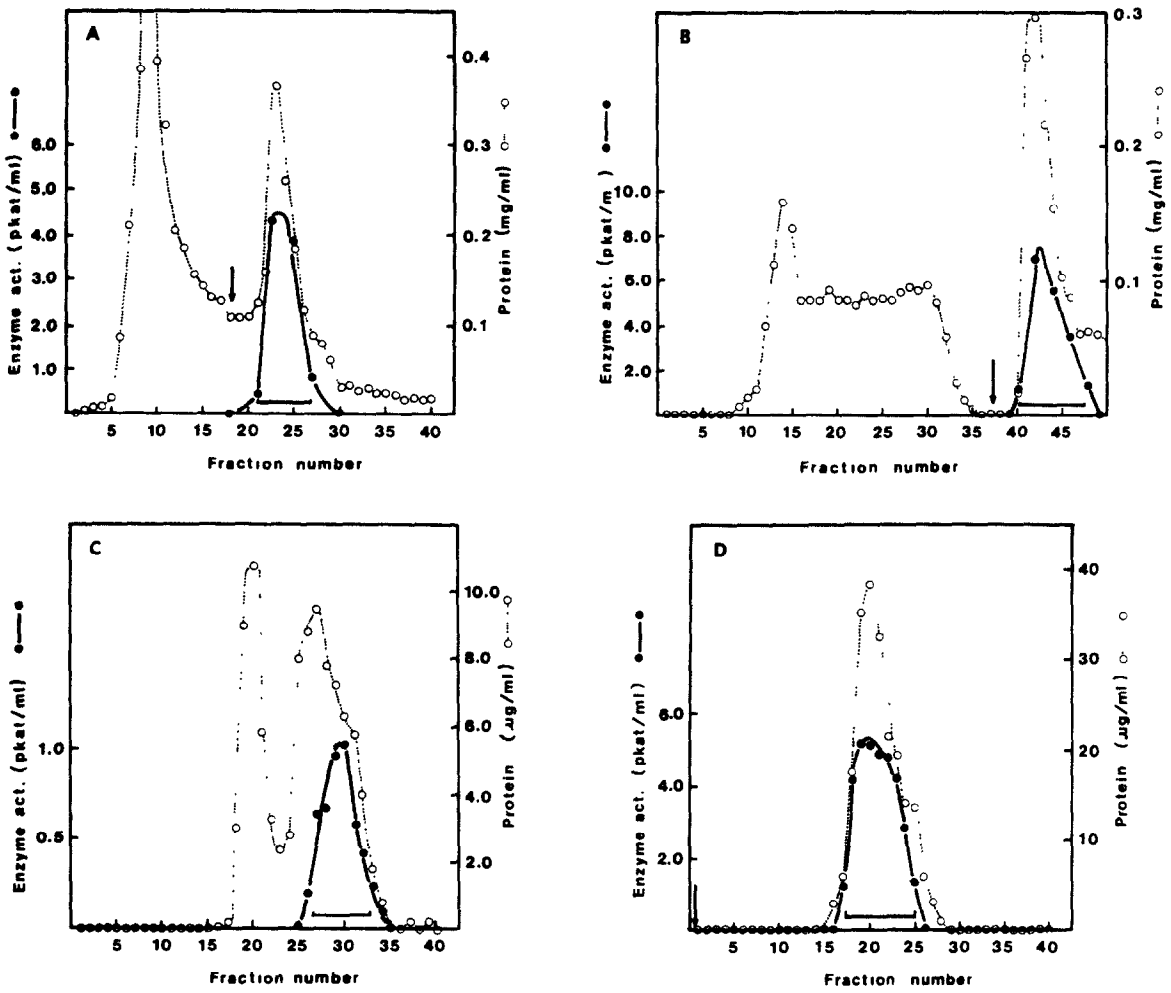

Fig. 5. (A) Phenyl-Sepharose CL-4B hydrophobic chromatography of 1,2-dehydroreticuline reductase preparation obtained after 60–85% ammonium sulphate precipitation. (B) DEAE-Sephacel anion exchange chromatography of pooled active fractions from step (A). (C) Ultrogel-AcA 34 gel filtration of the enzyme from DEAE-Sephacel step shown in (B). (D) Hydroxyapatite chromatography of the reductase preparation after passage through Ultrogel AcA 34 of step (C).

Table 1. Summary of the purification of 1,2-dehydroreticuline reductase from *Papaver somniferum* seedlings

Purification step	Volume (ml)	Protein (mg)	Total act. (pkat)	Spec. act. (pkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	180	336	51.1	0.15	100	1
$(NH_4)_2SO_4$ precipitation (60–85%)	10	47	34.5	0.74	68	5
Phenyl-Sepharose	35	6	78.5	13.4	154	88
DEAE-Sephacel	9	1.2	50.5	42.4	99	279
Ultrogel AcA 34	60	0.43	44.5	103	87	677
Hydroxyapatite	10	0.15	25.2	161	50	1062
FPLC-Superose 12	3	0.05	11.0	220	22	1447

Properties of 1,2-*dehydroreticuline reductase*

The M_r of the reductase was determined by employing a Pharmacia Superose 12 precalibrated gel filtration column. It was found that the reductase activity was eluted at a volume corresponding to a protein of 30000. Under denaturing conditions, the purified enzyme also showed a protein band at 30000 on a SDS polyacrylamide gel (Fig. 6). These results indicate that the reductase is a monomeric protein.

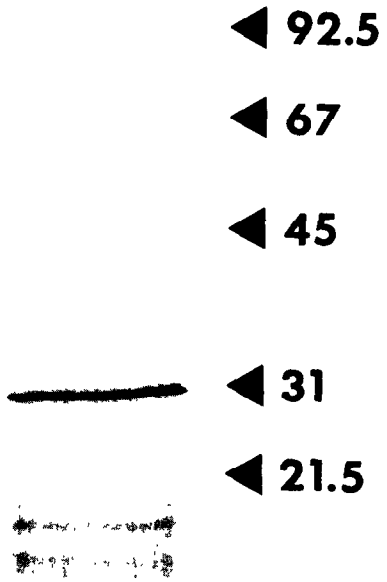

Fig. 6. SDS-polyacrylamide gel electrophoresis of the purified 1,2-dehydroreticuline reductase from *P. somniferum* seedlings. Arrows indicate the distribution of marker proteins and molecular mass ($\times 10^{-3}$).

The pH optimum of the enzyme was found to be 8.5. Under the conditions of the standard assay, the production of (*R*)-recticuline was linear with time for at least 45 min and proportional to the protein concentration. The optimal temperature for catalytic activity was determined to be 30°. Careful kinetic analysis revealed that for each mole of 1,2-dehydroreticuline consumed, 1 mol of (*R*)-reticuline is formed. The purified enzyme was stabilized by addition of NADPH (1 mM), showing half maximal activity after 20 days or 30 days when kept at 4° or −20°, respectively (Fig. 7). Omission of NADPH during the storage process affected rapid loss of activity with a half-life of two days or one day when stored at 4° or −20°, respectively.

NADPH is compulsory for catalytic turnover. No transformation of 1,2-dehydroreticuline was observed when NADPH was omitted. Also, replacement of NADPH with NADH rendered the enzyme completely inactive. When stereospecifically labelled coenzymes [4*R*-^{3}H]NADPH and [4*S*-^{3}H]NADPH were employed in the reaction mixture, it was found that radiolabel from only the [4*S*-^{3}H]NADPH species was transformed to the product (Fig. 8). The reductase, therefore, appears to be B-stereospecific and transfers the pro-*S*-hydride from NADPH to C-1 of the 1,2-dehydroreticulinium ion.

Substrate specificity and kinetic properties

The reductase enzyme appeared to exhibit high substrate specificity towards 1,2-dehydroreticuline. No reaction could be detected with closely related benzylisoquinolines such as 1,2-dehydronorreticuline and 1,2-dehydrococlaurine. The enzyme was also totally inactive towards salutaridine or codeinone. These two compounds are

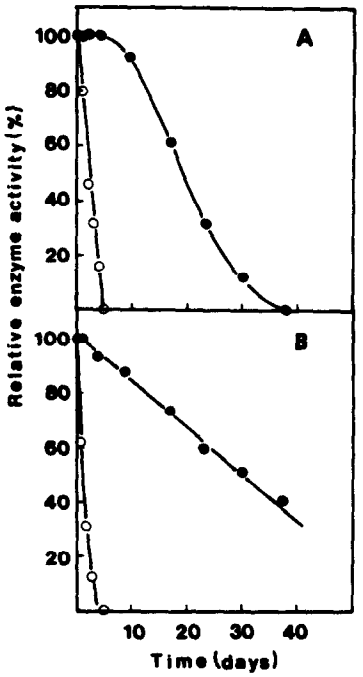

Fig. 7. Stability of the purified 1,2-dehydroreticuline reductase upon storage at 4° (A) and −20° (B) in the presence (●) and absence of (○) of 1 mM NADPH.

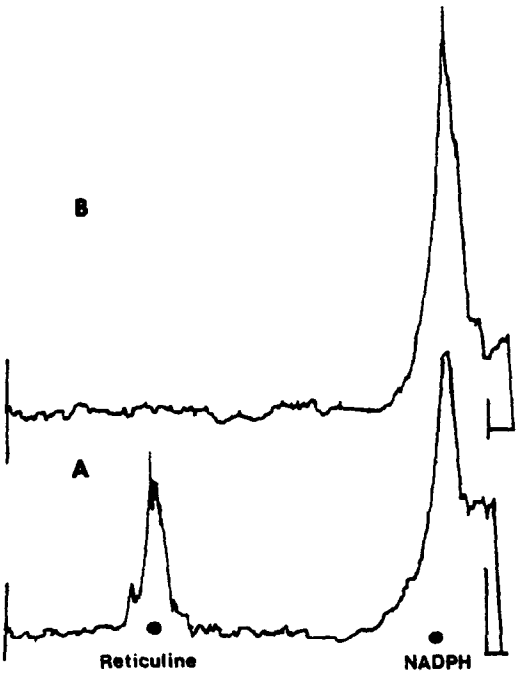

Fig. 8. Radioscans of complete standard assay mixture containing (A) 10 μM [4*S*-^{3}H]NADPH (6×10^5 dpm) and (B) 10 μM [4*R*-^{3}H]NADPH (6×10^5 dpm).

biosynthetic intermediates in the morphine pathway and are potentially enzymatically reduced to form salutaridinol I or codeine, respectively.

The K_m values for both 1,2-dehydroreticuline and NADPH were determined for the reductase enzyme. By keeping the NADPH concentration constant at 0.67 mM and varying 1,2-dehydroreticuline from 1.0 to 25 μM, the K_m for 1,2-dehydroreticuline was determined as 10 μM (Fig. 9). When the 1,2-dehydroreticuline concentration was fixed at 15 μM and the NADPH concentration varied from 0.01 to 0.67 mM, the K_m value for NADPH was determined to be 7 μM (Fig. 10).

Effect of biosynthetic intermediates of morphine on 1,2-dehydroreticuline reductase activity

Morphine and various potential precursors and intermediates in the morphinan pathway were tested for their

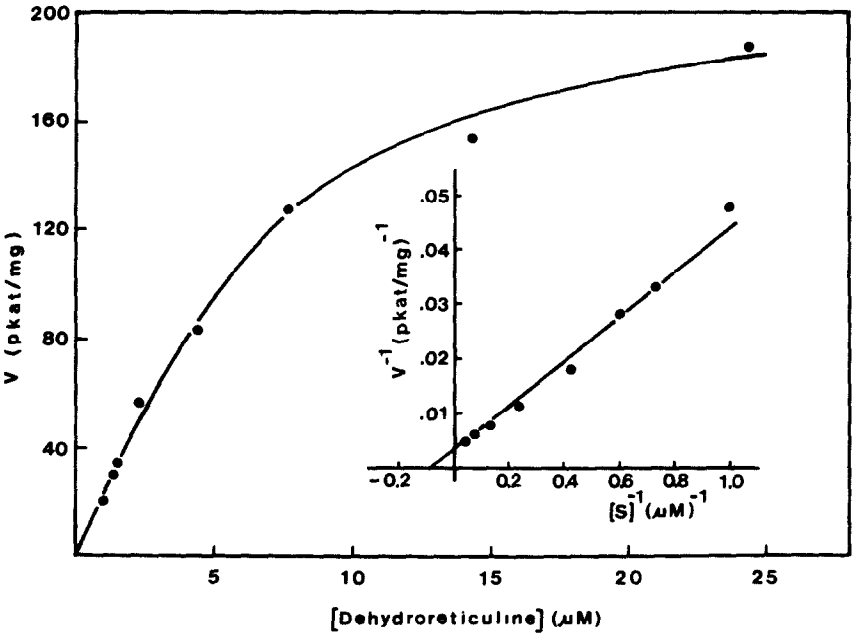

Fig. 9. The effect of 1,2-dehydroreticuline concentration on the activity of 1,2-dehydroreticuline reductase. The insert shows the double-reciprocal plot with $K_m = 10 \times 10^{-5}$ M.

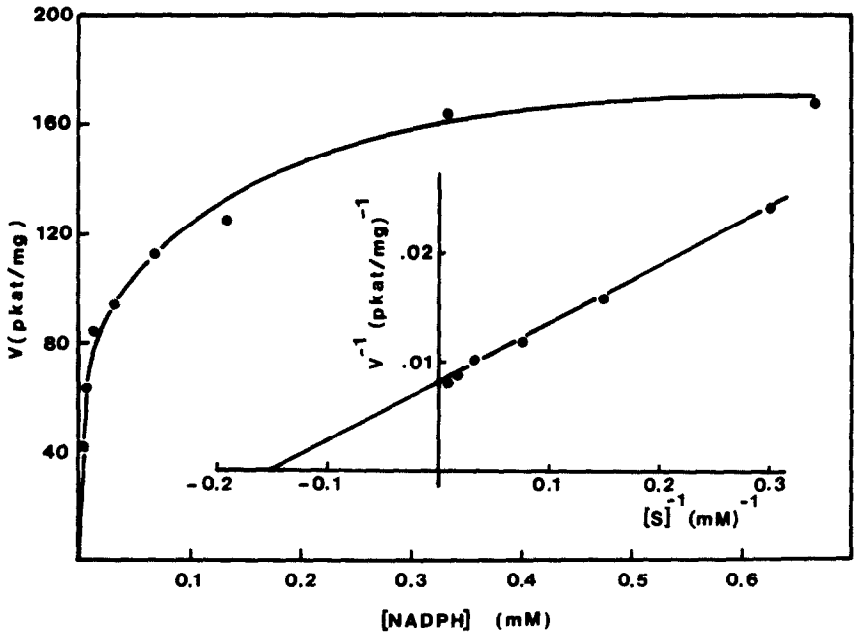

Fig. 10. The effect of NADPH concentration on the activity of 1,2-dehydroreticuline reductase. The insert shows the double reciprocal-plot with $K_m = 7 \times 10^{-5}$ M.

effects on reductase activity. Tyrosine, tyramine, DOPA, and dopamine have no effect on enzyme activity, 1-benzylisoquinoline and morphinan metabolites show various degrees of enzyme inhibition. Among these metabolites, (*S*)-reticuline, (*R*)-reticuline, 6-*O*-Me-laudanosoline and salutaridine appeared to effectively inhibit turnover. Their concentrations resulting in 50% inhibition were found to be 0.05, 0.10, 0.03 and 0.10 mM, respectively.

Occurrence of 1,2-*dehydroreticuline reductase*

The presence of 1,2-dehydroreticuline reductase in crude enzyme extracts of differentiated plants and cell cultures of some plant families was also investigated. Using the standard assay, the reductase was detected only in differentiated plants of *P. somniferum* and *P. bracteatum* which are known to contain morphinan alkaloids. No enzyme activity was detected in differentiated plants of *P. persicum, P. orcophilum, Argemone hunnemannii, Dicentra spectabilis* and *Fumaria macrosepta* (Table 3). Also, no activity was found in cell cultures of the families Papaveraceae, Berberidaceae and Ranunculaceae, all of which did not produce morphinandienone alkaloids. For subcellular enzyme localization in *P. somniferum* seedlings, it appeared that the reductase is a cytosolic protein. No indication was found for subcellular compartmentation using sucrose density (15–45%) gradient centrifugation (data not shown).

Table 2. Inhibition of 1,2-dehydroreticuline reductase by various biosynthetic intermediates of morphine

Compound	Inhibition (%) 0.1 mM	1.0 mM
Tyrosine	0	0
Tyramine	0	0
DOPA	0	0
Dopamine	0	0
(*S*)-Coclaurine	7	39
(*S*)-*N*-Me-Coclaurine	21	65
6-*O*-Me-Laudanosoline	75	99
(*S*)-Norreticuline	0	10
(*S*)-Reticuline	28	91
(*R*)-Reticuline	27	78
Salutaridine	52	85
Thebaine	27	82
Oripavine	21	60
Codeine	8	41
Morphine	5	16

Table 3. Occurrence of 1,2-dehydroreticuline reductase in different plants

Family	Species	Activity
Differentiated plants		
Papaveraceae	*Papaver somniferum*	20
	P. bracteatum	6.8
	P. persicum	0
	P. oreophilum	0
	Argemone hunnemannii	0
	Dicentra spectabilis	0
Fumariaceae	*Fumaria macrosepta*	0

DISCUSSION

Although the biosynthesis of morphinan alkaloids has essentially been clarified at the precursor feeding stage, little is known about their biosynthesis at the cell-free level. Among the nine largely hypothetical steps of the biosynthetic scheme [7] for the formation of morphine from (*S*)-reticuline, only two enzymic reactions have currently been demonstrated in a *Papaver somniferum* cell-free system. These enzymic reactions are the conversion of codienone to codeine [8] and the oxidative coupling of (*R*)-reticuline to salutaridine [9].

In this report, we have demonstrated one further enzymic reaction in the purported pathway, namely the reduction of the 1,2-dehydroreticulinium ion to form (*R*)-reticuline. A crucial point in this work was to unequivocally demonstrate that the product of this enzymic reaction is exclusively the (*R*)-form of reticuline. This was accomplished by employing a number of detection methods, including a (*R*)- and (*S*)-reticuline-specific radioimmunoassay, circular dichroism and an enzyme assay for (*S*)-reticuline with the berberine bridge enzyme. These results indisputably demonstrate the presence of a highly stereospecific biocatalyst in *Papaver somniferum* crude extracts.

By applying the enzyme radioassay developed in this study, we found a higher level of enzyme activity in the seedlings of *P. somniferum* as compared to those in the mature capsules, leaves, roots or cell cultures of the plant. Careful study of the enzyme activity during seed germination revealed that appreciated levels of activity appeared between days 4 and 7 after germination, this stage, therefore, serving as a source of the enzyme. Using the six-step purification procedure as described in this paper, it was possible to purify the enzyme to apparent electrophoretic homogeneity with 20% overall recovery and a specific activity varying from 170 to 220 pkat mg^{-1} protein. However, enzyme activity could only be stabilized for characterization by addition of NADPH (1 mM).

Characterization of the enzyme revealed an apparent molecular weight of 30 000, a pH optimum at 8.5 and a temperature optimum at 30°. Its activity was strictly dependent on NADPH. The enzyme exhibited a high specificity towards both the substrate 1,2-dehydroreticuline and the coenzyme NADPH with a relatively low affinity for both compounds. Absolutely no reduction was detected with 1,2-dehydronorreticuline or 1,2-dehydrococlaurine. These results support the original proposal [1] that the transformation of the (*S*)-benzylisoquinoline to the (*R*)-enantiomer takes place at the reticuline and not at the norreticuline level. Determination of the stereospecificity of the enzyme revealed that the reductase is of the B-stereospecific type. It transfers the pro-*S*-hydride from NADPH to C-1 of the iminium ion. We propose the name 1,2-dehydroreticuline reductase (NADPH) for this stereospecific novel enzyme.

The reason why enzyme activity is inhibited by both (*S*)- and (*R*)-reticuline is still unknown, as well as the observation that the degree of inhibition decreased in order going from salutaridine through the morphinan intermediates to morphine. *In vivo* experiments on the effects of these compounds on enzyme activity will have

to be pursued to ascertain the physiological significance of such inhibitions.

This novel enzyme was found to be a cytosolic protein and no indication was found for subcellular compartmentation using sucrose density (15–45%) gradient centrifugation. A survey for the reductase activity in both differentiated plants and cell cultures of some families showed that the enzyme was present only in morphinan-alkaloid containing plants. Among some members of the Papaveraceae tested, only *P. somniferum* and *P. bracteatum* displayed activity. In the cell cultures of both species which, however, did not produce these alkaloids, no enzyme activities could be detected.

There can be no doubt that this highly species- and substrate- as well as stereospecific enzyme catalyses the provision of (*R*)-reticuline for the formation of morphinandienone alkaloids also possessing the (*R*)-configuration at the corresponding chiral centre. The naturally occurring branch point intermediate 1,2-dehydroreticuline is specifically reduced as shown by *in vivo* [3] and *in vitro* (this study) experiments to (*R*)-reticuline, thus opening the pathway leading to the opium alkaloids.

EXPERIMENTAL

Plant material. The seeds of an inbred high alkaloid producing *Papaver somniferum* line were used. The seeds (1.0 g) were germinated in translucent plastic boxes (20 × 20 cm) containing 20 g vermiculite, 2 layers of tissue paper and 60 ml H_2O. Growth occurred under 20° and continuous light (1500 lux cool white fluorescent lamps). Whole plants of other species were grown either outdoors or in a greenhouse. All cell cultures were provided by the cell culture laboratory of this Department.

Chemicals. Tyrosine, tyramine, dopamine and DOPA were purchased from Fluka, Neu-Ulm. Codeine and morphine were purchased from Merck, Darmstadt; thebaine was from Boehringer, Ingelheim. Benzylisoquinolines and codeinone were synthesized according to standard procedures [10]. Optically pure (*S*)- and (*R*)-reticuline as well as (*S*)-norreticuline were obtained from Prof. N. Nagakura (Kobe). Salutaridine was synthesized according to ref. [11]. [4*S*-^{3}H]NADPH and [4*R*-^{3}H]NADPH were synthesized as described in ref. [12]. All biochemicals were obtained from Boehringer, Mannheim. Materials for chromatography were purchased from Pharmacia LKB, Uppsala (Sephadex G-25, Phenyl-Sepharose CL-4B, DEAE Sephacel, Superose 12, Ultrogel AcA 44) and Bio-Rad, Richmond (Hydroxyapatite). All other chemicals and solvents were purchased from Merck, Darmstadt or Roth, Karlsruhe.

Synthesis of (N^{14}CH$_3$)-1,2-*dehydroreticuline.* The method employed involved two enzymatic steps. First, [N-^{14}CH$_3$]-(*S*)-reticuline was synthesized from (*S*)-norreticuline using *S*-adenosyl-L-[^{14}CH$_3$]methionine (spec. act. 56 μCi μmol^{-1}) and a *N*-methyltransferase isolated from *Berberis stolonifera* cell cultures [13]. [N-^{14}CH$_3$]-1,2-Dehydroreticuline was then synthesized from radiolabelled (*S*)-reticuline as follows: a solution of [N-^{14}CH$_3$]-(*S*)-reticuline (10 μCi) was added to a reaction mixture containing, in a total vol. of 2 ml, 250 mM borate buffer pH 8.9, 0.1 mM phenanthroline, 10 mM ascorbate and 1 ml of partially purified STOX enzyme from *Berberis stolonifera* cell cultures (prepared by two chromatographic steps; DEAE cellulose and Ultrogel AcA 44 as described in ref. [14]). The reaction mixture was incubated for 48 hr at 30°. The mixture was then concentrated and the residue purified by TLC (Me_2CO–H_2O–Et_2NH, 8:1:1) to yield *ca* 5 μCi of (N-^{14}CH$_3$)-1,2-dehydroreticuline with a spec. act. similar to that of *S*-adenosyl-L-(^{14}CH$_3$)-methionine (56 μCi/μmol).

Synthesis of 1,2-*dehydroreticuline.* 1,2-Dehydroreticuline was synthesized from (*S*)-norreticuline in two steps as follows:

Step 1. A solution of (*S*)-norreticuline (0.6 mg) in H_2O was added to the reaction mixture containing, in a total vol. of 700 μl, 150 mM borate pH 8.9, 10 mM ascorbate and 500 μl of 0–70% $(NH_4)_2SO_4$ precipitated enzyme fraction from *Berberis stolonifera* cell cultures. Thirty-five tubes of the reaction mixture [a total of 20 mg of (*S*)-norreticuline] were prepared and incubated overnight at 30°. The mixture was pooled, freeze-dried and extracted with MeOH. After concn *in vacuo*, 1,2-dehydronorreticuline was purified by prep. TLC (CH_2Cl_2–MeOH–25% NH_3, 90:9:1; R_f 0.45). The sharp intense yellow band of dehydronorreticuline was eluted with MeOH resulting in *ca* 10 mg dried product.

Step 2. Purified 1,2-dehydronorreticuline was dissolved in 10 μl dry DMSO and 50 μl of MeI was added. The sealed reaction vessel was kept at room temp. in the dark for 24 hr. After evapn, the residue was chromatographed on silica with solvent system of Me_2CO–H_2O (8:1) to give *ca* 7 mg of 1,2-dehydroreticuline; MS, *m/z*: 327 $[M]^+$; UV λ_{max}^{MeOH} nm: 370, 310, 250.

Preparation of crude extracts for time-course study. All operations were carried at 0–4°. The freshly harvested seedlings (5 g) were ground in a cold mortar in 2 vols of 100 mM tricine–NaOH buffer pH 7.5 containing 250 mM sucrose, 1 mM EDTA and 10 mM 2-mercaptoethanol. The slurry was squeezed through four layers of cheesecloth and the filtrate centrifuged at 10 000 *g* for 10 min. The resulting supernatant (1 ml) (known volume) was desalted on a G-25 column (1.5 × 5.5 cm) and assayed for 1,2-dehydroreticuline reductase activity. Crude extracts of other plant sources or cell cultures were also prepared using the same procedure.

Enzyme preparation and purification. Deep-frozen 4- to 7-day-old seedlings (100 g) of *P. somniferum* were ground to a fine powder in a precooled mortar. The frozen powder was thawed and stirred (30 min) in 200 ml 100 mM tricine–NaOH buffer pH 7.5 containing 250 mM sucrose, 1 mM EDTA and 10 mM 2-mercaptoethanol (extraction buffer). The mixture was pressed through four layers of cheesecloth, and the filtrate was centrifuged at 10 000 *g* for 10 min. The supernatant was passed through an Amberlite XAD-2 column (3 × 20 cm). Finely ground solid $(NH_4)_2SO_4$ was added slowly to the eluate and the pellet that precipitated between 60 and 85% satn was collected by centrifugation (10 000 *g*, 10 min). The pellet was dissolved in a minimal vol. of extraction buffer supplemented with $(NH_4)_2SO_4$ to 5% satn (0.2 M). The concd enzyme soln (10 ml) was then applied to a Phenyl-Sepharose CL-4B column (1.3 × 16 cm) pre-equilibrated with the same buffer. After washing with 100 ml of the buffer at a flow rate of 1 ml min^{-1}, the column was eluted with 100 ml of extraction buffer and 5 ml frs were collected. Frs containing activities of 1,2-dehydroreticuline reductase were pooled (35 ml) and applied to a DEAE-Sephacel column (1.5 × 5.5 cm) pre-equilibrated with extraction buffer. The column was washed with 10 ml of the same buffer and the absorbed enzyme was eluted with 30 ml extraction buffer containing 5% saturation $(NH_4)_2SO_4$ (flow rate 1 ml min^{-1}, 1 ml per fraction). The active fractions were pooled (9 ml) and applied directly to a Ultrogel-AcA 34 gel filtration column (2.5 × 90 cm). The column was pre-equilibrated and eluted with 10 mM K-Pi, pH 7.5 containing 250 mM sucrose, 1 mM EDTA and 10 mM 2-mercaptoethanol (flow rate: 0.33 ml min^{-1}; fraction size: 10 ml). The pooled active fractions (60 ml) were loaded on to a hydroxyapatite column (1.3 × 7.5 cm) pre-equilibrated with the same buffer as used in the gel filtration step. After washing with 20 ml of the buffer at a flow rate of 1 ml min^{-1}, the column was eluted with 40 ml of 50 mM K-Pi, pH 7.5 containing 250 mM sucrose, 1 mM EDTA and 10 mM 2-mercaptoethanol while 1 ml frs were

collected. The active frs were pooled (10 ml) and concd to a volume of 1 ml by ultrafiltration (Amicon). The resulting preparation was finally subjected to FPLC-Superose 12 (HR 10/30, Pharmacia) gel filtration. The column was pre-equilibrated and eluted with 50 mM K-Pi, pH 7.5 (flow rate 0.4 ml min^{-1}; fraction size 1 ml). The fractions with the highest enzyme activity were pooled and used as the purified enzyme preparation.

Enzyme assay. The routine assay mixture contained, in a total vol. of 150 ml: 330 mM glycine–NaOH buffer, pH 8.5, 0.67 mM NADPH, 13.3 μM [N-$^{14}CH_3$]-1,2-dehydroreticuline (15000 dpm) and protein up to 0.1 mg. The reaction was terminated, after 30 min incubation at 30°, by the addition of 1 ml toluene. Tubes were vortexed for 20 sec to extract radioactive product into toluene and phase separation was achieved by centrifugation (all Eppendorf systems). The radioactivity of 0.75 ml of the organic layer was measured by scintillation counting.

Analytical procedures. For identification of reaction product, silica gel plates (Polygram SilG/UV 254; Macherey-Nagel) were used with the following solvent systems: (i) CH_2Cl_2–MeOH–25% NH_4OH (90:9:1), R_f 0.63, (ii) Me_2CO–$CHCl_3$–Et_2NH (7:2:1), R_f 0.54. Reticuline was quantified by a radioimmunoassay specifically directed towards each of the enantiomers [5].

The circular dichroism spectrum of reaction product was kindly recorded and interpreted by Prof. G. Snatzke (Bochum). The spectrum was recorded in MeCN (0.67 mM). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [15] with 11% gels. Protein concentration was determined by the method of Bradford [16] using BSA as a standard. Gel permeation chromatography for molecular mass analysis was done on a calibrated Superose 12 column (Pharmacia LKB). The column was equilibrated and run with 50 mM K-Pi, pH 7.5.

Time course of reaction-product formation. The reaction mixture contained in a total volume of 630 μl: 160 mM glycine–NaOH pH 8.5, 0.08 mM 1,2-dehydroreticuline, 0.32 mM NADPH and 75 μg (0.8 pkat) of 100-fold purified preparation ($(NH_4)_2SO_4$ fractionation and Phenyl-Sepharose chromatography). After incubation at 30° for 0.5, 1, 2, 3, 5 and 8 hr, each reaction mixture was frozen immediately in liquid N_2. After thawing, 5 μl from each mixture (triplicates) was removed for specific (*S*)- and (*R*)-reticuline radioimmunoassay as described in ref. [5].

Determination of the stereospecificity of 1,2-dehydroreticuline reductase. The stereospecificity of 1,2-dehydroreticuline reductase was determined as follows: a solution (120 μl) containing 400 mM glycine–NaOH, pH 8.5, 0.17 mM 1,2-dehydroreticuline, 10 μM [4*S*-^{3}H]NADPH or [4*R*-^{3}H]NADPH (6×10^5 dpm) and 5.4 μg purified enzyme. The mixture were incubated for 30 min at 30° and subjected to TLC (silica gel) using the solvent system of CH_2Cl_2–MeOH–25% NH_4OH (90:9:1). The distribution of radioactivity on the TLC plate was measured with a radioscanner.

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